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(54) Tripeptides with pharmacological properties

(57) Tripeptides having the following general formula:

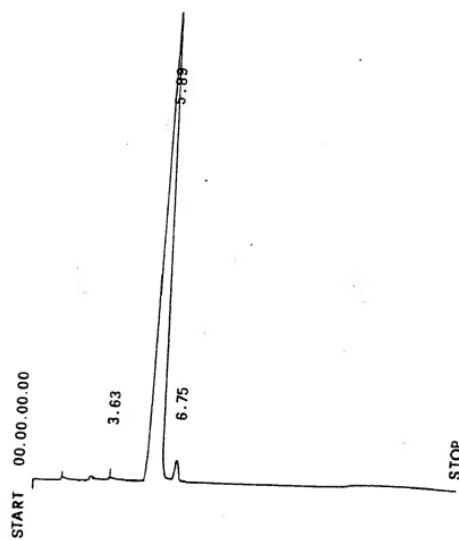


where X = L - Arg or D - Arg and Y = L - Asp or D - Asp, are disclosed, together with methods for the preparation thereof. These tripeptides, endowed with both immunostimulant and antimetastatic properties, are active not only after parenteral administration, but also after oral treatment.

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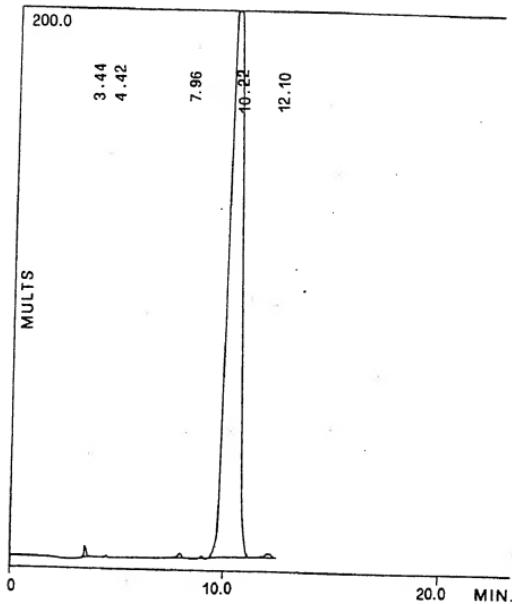
FIG.1: Profile HPLC di Arg-Gly-Asp



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FIG. 2: Profile HPLC di Arg-Gly-D-Asp



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TRIPEPTIDES AND PHARMACEUTICAL COMPOSITIONS CONTAINING THEM

This invention relates to tripeptides exhibiting interesting pharmacological properties, as well as to processes for their preparation and pharmaceutical compositions containing them.

It is known that the interaction between tumour cells and the extra-cellular matrix caused or promoted by fibronectin, is inhibited by intravenous administration of the synthetic pentapeptide Gly-Arg-Gly-Asp-Ser. The sequence for this peptide is the same as that of the cell-binding site of fibronectin (Humphries M.J. et al, Science 233:467, 1986). Ruoslahti and Pierschbacher (Cell 44:517, 1986) have suggested that the biological activity of this pentapeptide could be attributed to the tripeptide sequence Arg-Gly-Asp in the peptide but they had not tested the activity of the latter. Lam et al (J. Biol. Chem. 262:947, 1987) have instead shown that Arg-Gly-Asp is able to bind to some platelet surface glycoproteins, thus suggesting a possible biological action of this tripeptide as an inhibitor of platelet adhesion reactions.

An immunostimulating behaviour has been ascribed to the tripeptides: Arg-Lys-Glu (U.S. Patent Application No. 035,045 of April 6, 1987); Arg-Ala-Arg (U.S. Patent Application No. 035,044W of April 6, 1987); and Arg-Lys-Asp (European Patent Application No. 67425 of December 22, 1982).

We have now synthesized some tripeptides belonging to the general formula X-Gly-Y, where X is L-Arg or D-Arg and Y is L-Asp or D-Asp and have found that they exhibit interesting pharmacological activity.

Thus, in accordance with one aspect the invention provides a tripeptide of general formula:



(where X is L-Arg or D-Arg, and Y is L-Asp or D-Asp) and salts thereof with organic or inorganic acids.

In tests which we have carried out, the results have shown the efficacy of the tripeptides of the invention as antimetastatic agents. We have also found that the peptides surprisingly and unexpectedly exhibit a marked immunostimulating activity. The immunostimulating effect has been observed in vitro in experimental murine as well as in human models, and consists both in a maturation of immature T-lymphocytes and in an enhancement of T-cell function.

Moreover, we have found that the tripeptides of the present invention show stability in simulated gastric juice. Since it has been observed in the case of the peptides Arg-Lys-Glu and Arg-Ala-Arg that their stability in a simulated gastric environment is coupled with activity after oral administration to animals, we believe that it is possible to attribute to the tripeptides of the present invention an immunostimulating activity after oral, as well as parenteral, administration. This represents a remarkable advantage in their therapeutic use, particularly for children and other patients who do not tolerate parenteral administration, and also in view of the likelihood of better patient compliance.

Thus, the tripeptides according to the invention may be very useful in the prevention of metastases in neoplastic patients after surgical removal of a tumour, as well as for the simultaneous stimulation of the immune system. The latter effect may be very important in the post-operative treatment of neoplastic patients since their immune system may be depressed by both

radio/chemotherapeutic treatments and the operation itself: both of these factors being notoriously immunodepressant. Moreover, the ability of these drugs to be administered by an oral route represents, for the therapy of these patients, another undoubted advantage.

It will be appreciated that salts of the tripeptides according to the invention for use in medicine should be physiologically acceptable. Other salts may however be useful in the preparation of the peptides according to the invention or the physiologically acceptable salts thereof.

The invention also relates to the synthesis and the chemical as well as biological characteristics of the new class of tripeptides defined by the general formula X-Gly-Y, where X is L-Arg or D-Arg and Y is L-Asp or D-Asp.

The compounds according to the invention may be prepared by methods conventional to the art for synthesising peptides. Similarly, when a peptide is prepared as a salt thereof, it may be converted to other salts or to the free peptide according to methods known in the art.

In accordance with a further aspect of the invention there are provided pharmaceutical compositions comprising as active ingredient a tripeptide according to the invention or a physiologically acceptable salt thereof in association with a pharmaceutical carrier or excipient. The compositions according to the invention are preferably in a form suitable for oral or parenteral administration.

As indicated above, the compounds according to the invention are characterized by having antimetastatic properties (observed in tests of murine melanoma experimental metastases), as well as an

immunostimulating activity (shown by in vitro tests of murine spleen lymphocyte maturation (Thy 1.2 membrane marker induction), and in assays for the activation of human mature lymphocyte function (growth factor production, DNA and RNA synthesis after mitogenic stimulus, increase of mitoses)).

The products of the present invention are especially useful, thanks to their unique combination of antimetastatic and immunostimulating activities, as drugs in patients undergoing surgical tumour removal, in order to prevent metastasis formation, while at the same time helping to improve their immune status.

The following non-limiting Examples serve to illustrate the invention. In the Examples the following abbreviations are used:

Arg	= L-arginine
Gly	= glycine
Asp	= L-aspartic acid
Asx	= L-aspartic acid, D-aspartic acid or asparagine
D-Asp	= D-aspartic acid
Boc	= butyloxycarbonyl
N9	= substitution on the guanoisine nitrogen of Arg.

#### EXAMPLE I

##### Synthesis of L-Arginyl-glycyl-L-aspartic acid

###### 1. t-Butyloxycarbonyl-glycyl-L-aspartic acid dibenzyl ester

t-Butyloxycarbonyl-glycine (7.1 g) was dissolved in ethyl acetate (50 ml), cooled to -10 to -15°C with stirring and treated with N-methylmorpholine (5.6 ml) followed by isobutyl chloroformate (5.18 ml). The mixture was

stirred at -15°C for 10 minutes. Meanwhile, L-aspartic acid dibenzyl ester p-tosylate salt (20.1 g) was dissolved in dimethyl formamide (80 ml), cooled to -10°C and neutralized by adding N-methylmorpholine (5.6 ml). This solution was added to the above solution of the preformed mixed anhydride and the reaction mixture allowed to warm slowly to room temperature with stirring over 3 hours.

The reaction mixture was then diluted with ethyl acetate (300 ml) and the solution washed with brine (twice) followed by 4% aqueous sodium bicarbonate, water, 5% aqueous citric acid and finally with water to neutrality. The organic phase was dried (magnesium sulphate) and then evaporated to an oil. Yield: 20 g. The product was homogeneous by TLC (System: chloroform: methanol: acetic acid, 360:32:8;  $R_f = 0.8$ ).

2. Tribenzyloxycarbonyl-L-arginyl-glycyl-L-aspartic acid dibenzyl ester

The product from the previous step (20 g) was treated with 50% trifluoroacetic acid in methylene chloride (125 ml) for 25 minutes at room temperature and the solvent then evaporated under reduced pressure.

The residue was re-evaporated from toluene and then dried in vacuo. The resulting trifluoroacetate salt of the dipeptide was dissolved in dimethyl formamide (75 ml), cooled to -10°C and neutralized by treating with N-methylmorpholine (5.6 ml). Meanwhile, tribenzyloxycarbonyl-L-arginine (23.1 g) was converted to a mixed anhydride by dissolving in tetrahydrofuran (100 ml), cooling to -10 to 15°C and treating with N-methylmorpholine (5.6 ml) followed by isobutyl chloroformate (5.18 ml) as described above. The pre-cooled solution of the neutralized dipeptide was added to the solution of the mixed anhydride and the mixture stirred for 3 hours, warming slowly to room temperature. During the course of the

reaction, the mixture solidified and dimethyl formamide (50 ml) was added to facilitate stirring. The tetrahydrofuran was evaporated under reduced pressure and the residue diluted with ethyl acetate (500 ml),

This solution was washed with saturated aqueous sodium chloride, during which a white solid precipitated. This solid was filtered off, washed with ethyl acetate and dried to give the protected tripeptide. Yield: 25 g. Thin layer chromatography (System: chloroform:methanol:acetic acid, 85:10:5) showed slight contamination by unreacted tribenzylloxycarbonyl-arginine. The product (14 g) was therefore purified by dissolving in chloroform:methanol (98:2) and passing through a column of silica gel (600 g). The column was eluted with progressively increasing amounts of methanol in chloroform to give the pure protected tripeptide. Yield: 10 g.

3. L-arginyl-glycyl-L-aspartic acid

The protected tripeptide from the previous step (10 g) was hydrogenated at 350 kPa (50 psi) in methanol-acetic acid-water (60:20:20, 300 ml) for 72 hours over palladium on carbon (5%, 3 g), when the reaction was complete as shown by TLC. The catalyst was filtered off; the methanol evaporated under reduced pressure and the residue lyophilized to give the tripeptide as a white powder. Yield: 3 g. The product was homogeneous by thin layer chromatography (Systems: A, isopropanol:ammonia, 1:1; B, n-butanol-acetic acid:water:pyridine, 60:12:40:48).

Synthesis of L-arginyl-glycyl-D-aspartic acid

1. Preparation of Boc- $\beta$ -benzyl-D-aspartic acid-resins

Chloromethylated polystyrene (1% crosslinked; 200-400 mesh) was placed in a round-bottom flask and swollen in dimethylformamide (approximately

8-10 ml per gram of resin), then treated with Boc- $\beta$ -benzyl-D-aspartic acid (1 mmole per gram of resin), followed by potassium fluoride (2 mmole per gram of resin). The flask was equipped with a mechanical stirrer and condenser and heated under vacuum until a small amount (5-10 ml) of the solvent has distilled. The vacuum was removed and the mixture heated to 80-100°C for 16-18 hours. On cooling, the resin was filtered, washed with dimethylformamide, dimethylformamide:water (1:1), water, ethanol, dichloromethane and methanol, and then dried under vacuum. Substitution (as calculated by weight gain) = 9.6 mmole per gram.

2. Synthesis protocol

A quantity of 17.6 grams of Boc- $\beta$ -benzyl-D-aspartic acid-resin was placed in a glass reaction vessel equipped with a mechanical stirrer, a sintered glass base and a vacuum source for filtration. The resin was treated sequentially at ambient temperatures (20-25°C) with the following solvents or reagent:

- a.      methylene chloride
- b.      50% trifluoroacetic acid:methylene chloride (v/v)
- c.      50% trifluoroacetic acid:methylene chloride for 25 minutes.
- d.      methylene chloride (3 times)
- e.      isopropanol
- f.      10% triethylamine:methylene chloride (v/v) (twice)
- g.      methylene chloride
- h.      methanol (twice)
- i.      methylene chloride (twice)

A contact time of 3-5 minutes was allowed for each treatment. Approximately 10-15 ml of solvent or reagent-solvent mixture per gram of resin was used in each step.

j. The resin was stirred with a solution of Boc-glycine (3 equivalents) in methylene chloride, and to this was added dicyclohexylcarbodiimide (3 equivalents) in methylene chloride. The reaction time for coupling was a minimum of 2-4 hours but could be overnight (16-18 hours). The peptide-resin was filtered and washed with methylene chloride, methanol and methylene chloride and checked for completeness of coupling by the ninhydrin reaction. If coupling was incomplete, the same amino acid was recoupled using half the amount of reagents.

The cycle was repeated for Boc-*N*<sub>9</sub>-tosyl-L-arginine (in dimethylformamide). After removal of the N-terminal Boc-group, the resin peptide was washed thoroughly and dried under vacuum.  
Yield of resin - peptide : 20.1 g.

The peptide was cleaved from the resin and deprotected concomitantly by treatment with anhydrous liquid hydrogen fluoride, (approximately 10 ml per gram of resin-peptide) containing anisole (10% v/v) for 1 hour at 0°C. After evaporation of the hydrogen fluoride under reduced pressure, the crude peptide was extracted by washing the resin with dilute, aqueous acetic acid and the product isolated by lyophilization.  
Yield of crude peptide : 3.9 g.

### 3. Purification of the crude peptide

The crude peptide could be purified by preparative, reverse phase HPLC using C18-derivatized silica, using, for example, a Waters Prep 500 instrument. Using a 5 x 30 cm column, equilibrated with the appropriate aqueous buffer, such as 0.1% aqueous trifluoroacetic acid, the crude peptide (approximately 2 grams) was applied to the column and eluted with a gradient containing increasing amounts of acetonitrile. Fractions were monitored by analytical HPLC and those containing the product at the desired level of purity (>97%) were combined and lyophilized. Finally, the

purified product was converted to its desired salt by treatment with the desired salt form of an ion exchange resin.  
Yield of purified peptide as acetate salt: 2.5 g.

EXAMPLE 2: CHEMICAL CHARACTERISTICS

Aro-Gly-Asp

The data shown here are referred to a single batch of the tripeptide and should not be considered in a restrictive manner.

Molecular weight: 346.4

Appearance: white powder

Amino acid analysis

<u>Amino acid</u>	<u>Theory</u>	<u>Found</u>
Asx	1.00	1.00
Arg	1.00	0.98
Gly	1.00	1.02

Peptide content: 78.5%

Peptide purity: 96%

Humidity: 6.56

TLC: Isopropanol: NH<sub>3</sub> (1:1) purity 99%, R<sub>f</sub> = 0.75

HPLC: the analysis has been carried out according to the conditions hereafter described and the profile is shown in Figure 1 of the accompanying drawings.

Solvents: A = KH<sub>2</sub>PO<sub>4</sub> 0.05 M

B = 60% CH<sub>3</sub>CN + 40% A

Gradient: from 0 to 10% B in 20 minutes

Column: Ultrasphere ODS (ALtex)

Sensitivity: 0.2 AUFS

Wavelength: 210 nm

Flow rate: 1.5 ml/minute  
Minimum area: 10

Arg-Gly-D-Asp

The data shown here are referred to a single batch of the tripeptide and should not be considered in a restrictive manner.

Molecular weight: 346.4

Appearance: white powder

Amino acid analysis:

<u>Amino acid</u>	<u>Theory</u>	<u>Found</u>
Asx	1.00	0.94
Arg	1.00	1.05
Gly	1.00	1.02

Peptide content: 80.5%

Peptide purity: >98%

HPLC: the analysis has been carried out according to the conditions hereafter described and the profile is shown in Figure 2 of the accompanying drawings:

Solvents: A = NaH<sub>2</sub>PO<sub>4</sub> 25mM, 50nM NaClO<sub>4</sub>, pH 3.0 with H<sub>3</sub>PO<sub>4</sub>  
B = H<sub>2</sub>O:MeCN 1:1

Gradient: from 0 to 30% B in 15 minutes, linear  
from 30 to 70% B in 10 minutes, linear

Column: Deltapack C18 (5μm, 100 Å) 3.9 x 150 mm

Sensitivity: 0.2 AUFS

Wavelength: 220 nm

Flow rate: 0.7 ml/minute

Minimum area: 10

EXAMPLE 3: BIOLOGICAL CHARACTERISTICS

Stability in simulated gastric environment in vitro

The tripeptide Arg-Gly-Asp has been found to be stable at 37°C for 3 hours in a simulated gastric environment in vitro using a solution of simulated gastric juice USP XXI (HCl + pepsin).

Antimetastatic activity

The capacity of the tripeptide Arg-Gly-Asp to inhibit pulmonary colonization by B16-BL6 murine melanoma cells inoculated in female C57BL/6 mice having an average body weight of 20 g (10 animals per group) has been evaluated.

Arg-Gly-Asp was administered at a dose of 3 mg/mouse by intravenous injection together with  $2 \times 10^5$  melanoma cells.

Fourteen days later, the animals were sacrificed, the lungs were removed and examined, after formalin fixation, for the presence of surface melanoma colonies. A group of control animals were only treated with the tumour cell suspension.

While in the controls the mean number of metastases was above 500/mouse, this value was reduced in treated animals, where it was 173/mouse.

By administering  $7 \times 10^4$  tumour cells, the mean number of metastases in the controls was 21.6, which decreased to 6.8 in animals treated with 3 mg/mouse i.v. of Arg-Gly-Asp (-69%).

Induction of Thy 1.2 antigen in spleen cells of normal mice

The capacity of the tripeptide Arg-Gly-Asp to induce in vitro the

differentiation of T-cell precursors into lymphocytes expressing T-cell markers has been tested by evidencing the induction of Thy 1.2 membrane antigen.

#### CELL PREPARATION:

Spleen cells of normal mice were used as a source of "null" cells. Mice were killed by cervical dislocation. Spleens were aseptically removed and finely minced with forceps in Hank's balanced salt solution (HBSS) (Gibco Ltd, Paisley, Scotland). A suspension of single cells was obtained by filtration through a fine mesh wire sieve. Mononuclear cells (MNC) were isolated through differential centrifugation on Lymphoprep (Nyegaard, Oslo, Norway) with a continuous density gradient for 29 minutes at 450 g.

After 3 washes in HBSS, MNC were suspended at a concentration of  $5 \times 10^6$  cells/ml in 199 medium (Gibco Ltd) supplemented with 1% BSA (Cohn Fr. V. Sigma, St. Louis), L-glutamine 2mM and 10 mcg/ml of gentamycin sulfate (Schering, KIlworth, NJ, USA) (TC 199-BSA). The cells were used when their viability was over 95% in the Trypan Blue exclusion test.

#### THY 1.2 ANTIGEN INDUCTION BIOASSAY

Two hundred  $\mu$ l of the spleen MNC suspension were mixed with the same volume of the tripeptide diluted as required with TC 199-BSA medium. The control cells were treated with the TC 199-BSA medium. All the cells were incubated for 18 hours at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>, then washed twice with HBSS containing a 5% heat-inactivated neonatal calf serum (Gibco) (HBSS-CS) and finally re-suspended in the same medium at a concentration of  $10^6$  cells/ml.

#### DIRECT IMMUNOFLUORESCENCE

The expression of Thy 1.2 antigen was analysed by IF using an anti-Thy 1.2 monoclonal antibody conjugated with fluorescein (Bio-Yeda, supplied by

Technogenetics, S. Mauro Torinese, Italy) at a concentration of 2 mcg/10<sup>6</sup> cells. The cells were incubated with the antibody for 30 minutes at 4°C.

After three washes in HBSS, the cells were resuspended in the same medium and observed with a Leitz Orthomat microscope equipped with epi-illumination.

At least 300 cells were counted in each evaluation.

RESULTS:

As shown in Table I below, the tripeptide Arg-Gly-Asp is active *in vitro* in the induction of Thy 1.2 induction at concentrations ranging from 1 to 200 µg/ml, the optimum concentration being 10 mcg/ml.

TABLE I

ARG-GLY-ASP CONCENTRATION (µg/ml)	HOURS OF INCUBATION	% THY 1.2 + CELLS	VARIATION
-	3	22.0	-
0.1	3	25.3	+ 3.3
1	3	27.1	+ 5.1
10	3	31.4	+ 9.4
100	3	29.4	+ 7.4
200	3	27.9	+ 5.9
<hr/>			
-	16	17.0	-
10	18	25.5	+ 9.5
100	16	21.7	+ 4.7

RNA synthesis in PHA-stimulated human T-lymphocytes in vitro

Human T-lymphocytes, incubated in vitro for 24 hours in the presence of 0.5% phytohemagglutinin (PHA) and different concentrations of the test tripeptides, have been analysed for RNA synthesis (cell activation) by means of  $^{3}\text{H}$ -uridine labelling.

The results, shown in Table II below, show that both Arg-Gly-Asp and Arg-Gly-D-Asp are able to increase PHA-induced human T-lymphocyte activation.

TABLE II

PEPTIDE CONCENTRATION mcg/ml	ARG-GLY-ASP		ARG-GLY-D-ASP	
	c.p.m.		c.p.m.	
	$\bar{X} \pm S.E.$	$\Delta$ %	$\bar{X} \pm S.E.$	$\Delta$ %
0	3635	—	3635	—
	108	—	108	—
0.0001	3705	- 3	3517	- 6
	193	—	242	—
0.001	3741	- 2	4129	+ 8
	163	—	91	—
0.01	4259	+ 11	4283	+ 12
	191	—	114	—
0.1	4516	+ 18	4777	+ 25
	127	—	180	—
1	4055	+ 6	4682	+ 22
	278	—	456	—
10	4159	+ 9	4346	+ 13
	241	—	403	—

DNA synthesis in PHA-stimulated human T-lymphocytes in vitro

Human T-lymphocytes, incubated *in vitro* for 72 hours in the presence of 0.5% PHA and different concentrations of the test tripeptides, have been analysed for DNA synthesis (cell proliferation) by means of  $^{3}\text{H}$ -thymidine labelling.

The results, shown in Table III below, show that both Arg-Gly-Asp and Arg-Gly-D-Asp are able to increase PHA-induced human T-lymphocyte proliferation.

TABLE III

PEPTIDE CONCENTRATION mcg/ml	ARG-GLY-ASP		ARG-GLY-D-ASP	
	c.p.m.		c.p.m.	
	$\bar{x} \pm S.E.$	$\Delta \%$	$\bar{x} \pm S.E.$	$\Delta \%$
0	22642 3688	—	22642 3688	—
0.0001	22002 3583	+ 3	21898 3912	+ 3
0.001	23519 3712	+ 4	23029 3235	+ 2
0.01	24034 3625	+ 6	24551 3322	+ 8
0.1	25814 2931	+ 17	26020 3435	+ 15
1	25153 3805	+ 11	26170 4600	+ 16
10	24111 3537	+ 6	24570 4201	+ 9

Effect on cell cycle

Human T-lymphocytes were incubated for 72 hours in the presence of PHA and the test tripeptides (1 µg/ml). DNA has then been stained with propidium iodide and cells have been analysed with a flow cytometer. The results are set out in Table IV below.

TABLE IV

	CELL PHASES		
	G0-G1		S
	% CELLS	% CELLS	% CELLS
T + PHA	63.36	-	30.61
T + PHA + ARG-GLY-ASP	57.69	-5.67	29.46
T + PHA + ARG-LYS-ASP	60.79	-2.57	30.63
T + PHA + ARG-LYS-GLU	58.15	-7.21	28.64

G0 = resting cells

G1 = interval between mitosis and DNA synthesis

S = DNA synthesis

G2 = interval between DNA synthesis and mytosis

M = mytosis

Stimulation of lymphokine production in vitro

Human T-lymphocytes were incubated with PHA with or without the peptides under examination for 24 hours (in the case of IL-2) or 72 hours (in the case of BCGF).

The supernatants were collected, filtered ( $0.2 \mu\text{m}$ ) and assayed for the presence of IL-2 or BCGF activity, by adding them at different concentrations to fresh T-lymphocytes or to long term cultured B cells. The proliferating activity of these cells, dependent on the presence of the respective growth factor, was evaluated through  $^3\text{H}$ -thymidine incorporation.

The results show a marked effect on the stimulation of growth factor production by Arg-Gly-Asp, slightly lower than that of Arg-Lys-Glu, but higher than that obtained with Arg-Lys-Asp.

BICP ACTIVITY (COUNTS PER MINUTE) AT THE PERCENTAGE OF SUPERNATANT:

SUPERNATANT FRACTION:	N° of dilution	3.125			6.25			12.5			25			50		
		$\bar{X}$	S.E.	$\Delta\%$												
T + PIA	2	1345	--		2156	--		5402	--		15030	--		17297	--	
		627			193			290			294			1650		
T + PIA + MG-LYS-GLU	2	5377	+300		10125	+370		18143	+236		21977	+46		22666	+31	- 18 -
		2459			4503			3006			2702			3930		
T + PIA + MG-GLY-ASP	2	4800	+257		6611	+299		16550	+206		18645	+24		20506	+19	
		2397			3075			2312			2469			368		
T + PIA + MG-LYS-ASP	2	3654	+169		6455	+199		12219	+126		16554	+10		16107	- 7	
		1706			2320			1346			4076			1629		

## TGF ACTIVITY

T + PHA	2	1564	—	2622	—	4012	—	7024	—	14086	—
		329	—	643	—	1136	—	2657	—	3241	—
T + PHA	2	4516	+169	10213	+290	16609	+314	20695	+165	16451	+31
+ Arg-Lys-Glu		1320		4526		6126		5001		2176	
T + PHA	2	3245	+107	8496	+224	13113	+227	16609	+105	18167	+29
+ Arg-Lys-Asp		470		4359		4249		6126		3231	
T + PHA	2	3478	+122	8139	+210	13977	+240	15911	+103	16476	+17
+ Arg-Lys-Asp		1340		4253		6492		5237		5899	

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Stimulation of lymphokine in vitro by pure human T-lymphocytes, without  
macrophages

Lymphokine production by human T-lymphocytes purified by passage on a nylon wool column was evaluated with the same methods as in the previous experiment.

T-cell purity was above 95%, while macrophages/monocytes as well as B-cells were under 1%. In order to obviate the lack of macrophages, recombinant IL-1  $\beta$  (Genzyme) was added at a concentration of 25 Units/ml.

The results obtained, both for IL-2 and BCGF, show that the effect of Arg-Gly-Asp is comparable with that of the other reference compounds, among which thymosin fraction 5, a partially purified thymic derivative, was also used.

BCDF ACTIVITY AT THE PERCENTAGE OF SUPERNATANT:

Supernatant from:	3.125	6.25	12.5	25	50
	c.p.m.	c.p.m.	c.p.m.	c.p.m.	c.p.m.
T <sub>1</sub> PHA, T <sub>1</sub> -1	318	—	502	—	1104
T <sub>1</sub> (PHA+II,-1+ARG-LYS-Glu) (1mcg/ml)	319	+ 10	564	—	2866
T <sub>1</sub> (PHA, II,-1+ARG-LYS-Glu) (10 mcg/ml)	622	+ 96	784	+ 35	3196
T <sub>1</sub> (PHA, II,-1+ARG-Ala-Lys-Lys) (1mcg/ml)	496	+ 56	516	—	113
T <sub>1</sub> (PHA, II,-1+ARG-Ala-Lys-Lys) (10mcg/ml)	312	—	425	—	2347
T <sub>1</sub> (PHA+II,-1+ARG-Gly-Asp) (1mcg/ml)	618	+ 94	634	+ 9	2663
T <sub>1</sub> (PHA+II,-1+ARG-Gly-Asp) (10mcg/ml)	784	+47	866	+ 49	2897
T <sub>1</sub> PHA, II,-1+ARG-LYS-Asp (1mcg/ml)	326	+ 3	370	—	1039
T <sub>1</sub> PHA, II,-1+ARG-LYS-Asp (10mcg/ml)	421	+ 32	462	—	1847
T <sub>1</sub> (PHA, II,-1+THYMOS, PS) (10mcg/ml)	410	+ 31	921	+ 50	1796
T <sub>1</sub> (PHA, II,-1+THYMOS, PS) (20mcg/ml)	716	+125	566	—	1308

## TCGF ACTIVITY

Supernatant from:	TCGF ACTIVITY											
	3.125	6.25	12.5	25	50							
c.p.m.	Δ %	c.p.m.	Δ %	c.p.m.	Δ %	c.p.m.	Δ %	c.p.m.	Δ %	c.p.m.	Δ %	
T <sub>1</sub> PhA <sub>1</sub> I <sub>1</sub> -1	1696	---	2817	---	3663	---	4812	---	6287	---		
T <sub>1</sub> PhA <sub>1</sub> I <sub>1</sub> -1+MIG-LYS-Glu (1mcg/ml)	2516	+ 48	8216	+192	14932	+308	17148	+256	16293	+159		
T <sub>1</sub> PhA <sub>1</sub> I <sub>1</sub> -1+MIG-LYS-Glu (10 mcg/ml)	2873	+ 69	7192	+155	15264	+317	16887	+251	12246	+ 94		
T <sub>1</sub> PhA <sub>1</sub> I <sub>1</sub> -1+MIG-Ala-Ala (1mcg/ml)	2085	+ 23	5679	+102	11728	+220	12692	+164	11378	+ 81		
T <sub>1</sub> PhA <sub>1</sub> I <sub>1</sub> -1+MIG-Ala-Ala (10mcg/ml)	3142	+ 85	6447	+129	13629	+272	15143	+215	10875	+ 73		
T <sub>1</sub> PhA <sub>1</sub> I <sub>1</sub> -1+Ala-Ala-Gly-Asp (1mcg/ml)	1984	+ 17	6893	+145	13147	+259	14862	+209	15654	+149		
T <sub>1</sub> PhA <sub>1</sub> I <sub>1</sub> -1+MIG-Gly-Asp (10mcg/ml)	1736	+ 2	6342	+125	13874	+279	10968	+128	13427	+113		
T <sub>1</sub> PhA <sub>1</sub> I <sub>1</sub> -1+MIG-LYS-ASP (1mcg/ml)	2274	+ 34	5216	+ 85	8969	+145	10421	+117	9887	+ 57		
T <sub>1</sub> PhA <sub>1</sub> I <sub>1</sub> -1+MIG-LYS-Asp (10mcg/ml)	2769	+ 43	6143	+110	7245	+ 98	7963	+ 65	8143	+ 29		
T <sub>1</sub> PhA <sub>1</sub> I <sub>1</sub> -1+TIVYVMS-PS (10fmole/ml)	2374	+ 40	7206	+159	15637	+327	13142	+173	13035	+120		
T <sub>1</sub> PhA <sub>1</sub> I <sub>1</sub> -1+TIVYVMS-PS (20fmole/ml)	2156	+ 27	5218	+ 85	12143	+232	10874	+126	12130	+ 93		

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EXAMPLE 4: TOXICOLOGICAL TRIALS

Arg-Gly-Asp

The tripeptide Arg-Gly-Asp shows an LD<sub>50</sub> greater than 1000 mg/kg i.p. in mice.

EXAMPLE 5: CLINICAL USE

The previous Examples 3 and 4 have shown that the tripeptides of the present invention are active in vitro as immunostimulating agents in both animal and human experimental models, and in vitro as antimetastatic products in laboratory animals, as well as being substantially devoid of toxicity.

Thus, it can be predicted very reasonably that they will be clinically useful in preventing metastases in patients undergoing surgical tumour removal, at the same time improving the immune defences of the patient thanks to their immunostimulating properties.

SALTS OF THE TRipePTIDES

The above mentioned examples refer to the use of the acetate salts of the tripeptides. However, it is possible to obtain analogous results with other salts of organic and inorganic acids, such as, for example, their trifluoroacetate, hydrochloride or sulfate salts.

CLAIMS

1. A tripeptide of general formula:

X - Gly - Y

(where X is L-Arg or D-Arg, and Y is L-Asp or D-Asp) and salts thereof with organic or inorganic acids.

2. An acetate, trifluoroacetate, hydrochloride or sulfate salt of a tripeptide as defined in claim 1.

3. A tripeptide according to claim 1, having the sequence:

Arg-Gly-Asp

D-Arg-Gly-Asp

Arg-Gly-D-Asp

D-Arg-Gly-D-Asp

and salts thereof.

4. A tripeptide or formula Arg-Gly-Asp or Arg-Gly-D-Asp, and salts thereof.

5. A compound according to claim 1 as herein specifically disclosed.

6. Use of a compound according to any preceding claim, for the preparation of a medicament for use as an antimetastatic agent.

7. Use of a compound according to any one of claims 1 to 5 for the preparation of a medicament for use as an immunostimulating agent.

8. Use according to either of claims 6 and 7 for the preparation of a medicament for parenteral administration.

9. Use according to either of claims 6 and 7 for the preparation of a medicament for oral administration.
10. A pharmaceutical composition comprising as active ingredient a compound according to any one of claims 1 to 5 in association with a pharmaceutical carrier or excipient.
11. A pharmaceutical composition according to claim 10 in a form suitable for parenteral administration.
12. A pharmaceutical composition according to claim 10 in a form suitable for oral administration.
13. Use of a tripeptide or a pharmaceutically acceptable salt thereof according to any one of claims 1 to 4 for the preparation of a medicament for the treatment of a patient undergoing surgical tumour removal in order to prevent the formation of metastases and at the same time contribute to the improvement of the patient's immune condition.
14. Use according to claim 13 of an acetate salt.
15. Use according to claim 13 of a trifluoroacetate salt.
16. Use according to claim 13 of a hydrochloride salt.
17. Use according to claim 13 of a sulfate salt.
18. A process for the preparation of a compound according to any one of claims 1 to 5 which comprises: preparing t-butyloxycarbonyl-Gly-Asp dibenzyl ester, subsequently preparing the tribenzyloxycarbonyl-Arg-Gly-Asp dibenzyl ester, and subsequently catalytically hydrogenating the latter

latter ester to obtain the desired tripeptide or salt thereof.

19. A process for the preparation of a compound according to any one of claims 1 to 5 substantially as herein described in any one of the Examples.
20. Use according to any one of claims 6 to 9 substantially as herein disclosed.
21. A composition according to any one of claims 10 to 12 substantially as herein disclosed.
22. A process according to claim 18 substantially as herein disclosed.
23. Each and every novel product, process, method, composition, feature and combination of features substantially as herein disclosed.